ALTERED GENE EXPRESSION PROFILES OF RAT LUNG IN RESPONSE TO AN EMISSION PARTICULATE AND ITS METAL CONSTITUENTS

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Running title: Gene expression profile in PM-induced lung injury

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Abstract

Comprehensive and systematic approaches are needed to understand the molecular basis for the health effects of particulate matter (PM) reported in epidemiological studies. Due to the complex nature of the pollutant and the altered physiological conditions of predisposed populations, it has been difficult to establish a direct cause and effect relationship. A high throughput technology such as gene expression profiling may be useful in identifying molecular networks implicated in the health effects of PM and its causative constituents. Differential gene expression profiles derived for rat lungs exposed to PM and its constituent metals using a custom rat cardiopulmonary cDNA array are presented here. This array consists of 84 cardiopulmonary-related genes representing various biological functions such as lung injury/inflammation, repair/remodeling, structural and matrix alterations, and vascular contractility as well as 6 expressed sequence tags (ESTs). The cDNA array was hybridized with ^{32}P-labeled cDNA generated from rat lung RNA. Total lung RNA was isolated from male Sprague Dawley rats at 3 and 24 h following intratracheal instillation of either saline, residual oil fly ash (ROFA; 3.3 mg/kg) or its most toxic metallic constituents: nickel (Ni) ($NiSO_4$; 3.3 μmol/kg) and vanadium (V) (VSO_4; 5.7 μmol/kg). Metal concentrations reflected the levels present in one ROFA instillate. Densitometric scans of the array blots indicated ROFA and metal specific increased expression (1.5-3 fold) of stress response, inflammatory, and repair-related genes, and also genes involved in vascular contractility and thrombogenic activity. Expression of multiple cytokines in ROFA exposed rat lung compared to Ni and V suggest the role and importance of understanding constituent

interactions in PM toxicity. Expression profiling using genomic approaches will aid in our understanding of toxicant-specific altered molecular pathways in lung injury and pathogenesis.

Key words: particulate matter, cardiopulmonary, genearray, expression.

Introduction

Epidemiological studies have associated adverse health effects and exposure to air pollutants, including ambient particulate matter (PM). Increased mortality and morbidity reported in susceptible populations during high air pollution episodes suggest a relationship between PM air pollution and impairments of the cardiopulmonary function (Pope et al.,1992, Burnett et al., 1995, Dockery et al., 1993, MacNee and Donaldson, 2000). The underlying biochemical and physiological changes in cardiac and pulmonary tissues and the complex nature of PM (Bouthillier et al.,1998, Becker and Soukup, 1999, Hamada et al., 1999) obscure efforts to establish a direct cause and effect relationship. The need for mechanistic understanding of these relationships necessitates more comprehensive and systematic approaches to understand perturbations of basic cellular and molecular organizations. Studies carried out using laboratory animals and cells harvested from human bronchoalveolar lavage fluid have provided basic toxicity data for certain ambient PM (Imrich et al., 2000, Frampton et al., 2000), surrogate PM, residual oil fly ash (ROFA) and its constituents (Dreher et al., 1997, Broeckaert et al., 1997, Kodavanti et al., 1997, 1998a). A detailed and systematic approach towards understanding the molecular basis for the deleterious effects of PM and its constituents requires a high throughput technology to identify constituent-specific alterations and their potential interactions.

With the completion of human genome sequencing and the advancement in the technology for gene expression analysis, such as DNA microarrays, reverse imaging, and amplified fragment length polymorphism (AFLP), it is now possible to derive

comprehensive mRNA expression profiles (DeRisi et al., 1996, Bowtell, 1999). With the technological strides in large scale cloning enterprises in the industrial sector, microarrays have become the most common choice for gene expression analysis. Most of the commercial microarrays contain thousands of genes that can be screened in a one step hybridization. The generation of total gene expression profiles using microarrays of ~10,000 genes is no doubt a comprehensive approach. However, to ascertain the relevance of these data to assess the progress of disease or to determine mechanistic and predictive toxicological end points for pharmaceuticals and environmental toxicants, may favor a more focused approach (Pennie et al., 2000, Stanton et al., 2000). In fact studies carried out using microarrays containing ~5000 and more genes (Stanton et al., 2000, McDowell et al., 2000) indicated altered expression in only 1-2% of target genes that appear to be critical for the biological processes under investigation. Developing custom genearrays, with a choice of genes selected and clustered together, based on specific signaling pathways or pathophysiological processes (Sehl et al., 2000), would provide a focused, meaningful expression profile and would aid in the generation of new hypotheses.

The proinflammatory cytokines/chemokines, reactive oxygen and nitrogen species and lipid mediators released by pulmonary epithelial cells and macrophages in response to airborne pollutants, or allergens, and subsequent interactions with other cell types in the lungs can initiate a cascade of pathobiological processes (i.e., inflammation, repair and remodeling) (Martin et al., 1997). These coincidental and evolving events are amenable to evaluations via temporal expression profiles of genes

mediating the overall pathophysiological processes. With this aim a rat cardiopulmonary genearray was developed containing 84 selected genes that play critical roles in pulmonary and cardiac physiology and disease pathogenesis. These 84 genes are grouped into 7 functional gene clusters representing various biological processes. The details on selection, cloning, design and development of the genearray have been reported earlier (Nadadur et al., 2000). Data presented include the gene expression profile derived for rat lungs exposed to ROFA and two major toxic metallic constituents, nickel (Ni) and vanadium (V) in an effort to understand possible molecular mechanism(s) of action for analagous complex air pollutant like ambient PM.

Methods

Animals:

Sixty-day old, male Sprague Dawley (SD) rats weighing 250-300 g were obtained from Charles River Laboratories (Raleigh, NC) and housed in an AAALAC approved animal facility (21 ± 1^{o} C, 50 ± 5% RH, 12:12 h light/dark cycle). All animals were quarantined for one week and received standard Purina rat chow (Brentwood, MO) and water *ad libitum*.

Intratracheal (IT) Instillation:

Combustion source residual oil fly ash (ROFA), with a mass median aerodynamic diameter of 1.95 μm and a geometric standard deviation ($ó_g$) of 2.14 (Dreher, et al., 1997) was suspended in pyrogen-free bacteriostatic saline (3.3 mg/ml) and mixed gently for 20 min. V and Ni concentrations (two predominant toxic metals in ROFA) in one ml of ROFA instillate were calculated based on our previous study (Kodavanti et al., 1997) and accordingly nickel sulfate (NiSO4; 1.3 μmol/ml) and vanadium sulfate (VSO4; 2.2 μmol/ml) solutions were prepared in saline. Acidified saline (because ROFA suspension in saline resulted in pH of ~2.5), ROFA, V or Ni suspensions were IT instilled in rats (1 ml/kg body weight) under halothane anesthesia (Costa et al., 1986).

RNA extraction :

Three or 24 hours following IT instillation, rats were anesthetized with sodium phenobarbital (Nembutal, Abbott Lab., Chicago; 50-100 mg/kg body weight, intraperitoneal) and exsanguinated via the abdominal aorta. The right lung lobes were

ligated and immediately frozen in liquid nitrogen and stored at -80 ^{o}C for RNA extraction. RNA was isolated from frozen right lung lobes using Trizol reagent (GIBCO-BRL, Grand Island, NY).

Cloning of PCR-derived cDNAs:

Eighty four selected genes, including a number of constitutively and abundantly expressed genes were cloned as described earlier (Nadadur et al., 2000). The size and the authenticity of cDNA inserts were confirmed by restriction digestion analysis, DNA sequencing and BLAST homology search (Pearson and Lipman, 1988).

Array Blotting:

Plasmid DNA (100 ng) for the respective clones was denatured by alkali exposure and blotted onto GeneScreen plus membranes (NEN-DuPont, Boston, MA) using a Bio-Rad Dot blot apparatus according to the manufacturer's recommendations. The membranes were then baked at 80^{o} C for 2 h under vacuum and stored at room temperature until used.

The template indicating the spot locations on the array blot, in a 96 well format containing 84 cardiopulmonary function specific genes, 6 ESTs (4 mouse and 2 human) two spots with cloning vector DNA and 4 blank spots is presented in Fig.1. Vector DNA and blank spots were used to monitor non specific hybridization, while β-actin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), the two house-keeping genes served as positive controls.

Hybridization:

Lung total RNA (15 μg) from control, ROFA, Ni or V exposed rats (n = 4; RNA

pooled from 2 animals and samples run in duplicate) was reverse transcribed using Superscript II reverse transcriptase in a reaction mix containing ^{32}P-dATP and random hexamers as detailed earlier (Nadadur et. al., 2000) with the following modifications. The reverse transcription reaction was extended for 15 min with 0.1mM cold dATP to avoid the formation of truncated cDNAs. The labeled cDNA produced in the reverse transcription reactions was purified using Chromaspin-200 column (Clonetech, Palo Alto, CA). The pooled fractions containing the labeled probe were denatured with alkali exposure, neutralized with sodium phosphate buffer, and then added to the hybridization mix. Each hybridization mix containing separate cDNA probes (from lung RNA of control, ROFA, Ni or V -exposed rats) were incubated in parallel with array membranes. The membranes were submerged in hybridization buffer and hybridization was carried out overnight at 42^{o} C. All blots were subjected to high stringency wash until the background radioactivity was minimal. The washed blots were either exposed to X-ray film for 15-30 h or to Phosphor screen for 3-4h and scanned using Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The density of the spots on the scan images was quantitated using an Imagequant software program (Molecular Dynamics, Sunnyvale, CA).

RT-PCR :

Pooled total lung RNA (5 μg) from control, ROFA, Ni or V-exposed rats was reverse transcribed using random hexamers and amplified in a PCR for IL-6, E-selectin, TIMP-2, SP-A and β-actin using gene specific primers. PCR amplified gene fragments were separated by electrophoresis through 1.5 % agarose gel (Nuseive 3:1) containing

ethedium bromide. Photographic images of the gel was scanned and the intensity of DNA bands were densitometrically quantitated.

Genearray data analysis:

Phosphorimages and scanned autoradiograms were analyzed using Imagequant software program. Density units obtained for blank spots were subtracted from the DNA spots after adjusting for background correction carried out using local average parameter. Additionally, density units for each spot was normalized to β-actin in that blot. The final density values derived for each spot after the normalization was used to calculate the ratio for spot density of experimental compared to control. Difference in the spot density above 1.5 fold was used to identify any change as a real change in gene expression. Two array blots were hybridized for each group.

Results:

Detection of differentially expressed cDNAs :

A rat cardiopulmonary cDNA genearray containing 84 selected genes grouped into 7 functional gene clusters (Table 1) were blotted on to the nylon membrane following the template presented in Fig.1. ^{32}P-labeled cDNAs generated in the reverse transcriptase reaction using RNA from control (saline exposed), ROFA, Ni-, and V-exposed rat lungs were hybridized in parallel to the array blots as detailed in the methods section. Representative scan images of the array blots hybridized with probes generated from the 3 and 24 h post exposure groups are represented in Figs. 2 and 3, respectively and the data for fold change in gene expression in response to exposures are presented in Table 2. Strong and intense hybridization signals detected for a group of transcripts, such as NGF, cyclin E and EST-3 (a novel EST isolated from transformed mouse cell line) (Nadadur et al., 1997) indicate high basal expression of these genes in the lung.

Array hybridization data presented in Figs 2 and 3 and Table 2 indicate constituent specific and time-dependent altered gene expression profile in the rat lung tissue exposed to ROFA and its constituent metals. Three groups of genes: inflammation, remodeling, and stress-response were found to be induced in response to ROFA, Ni and V exposure. ROFA-induced expression of PDGF-A, TGF-β, E-selectin and TIMP-2, were observed at 3 and 24 h post exposure and the fold induction was similar for both time points studied. Increased expression of NOS, NOS(I) and MAPP observed at 3h post ROFA exposure returned to basal levels by 24h. ROFA-induced

expression of the cytokines, IL-5, IL-6, RANTES and the adhesion molecule ICAM-1 were observed at 24 h post exposure (Fig. 3, Table 2).

Ni-induced rat lung injury appeared to involve the expression of genes involved in stress response (HO-2, hsp70, TIMP-2) and cell adhesion (E-selectin, C-Fn, ICAM-1). Ni-induced overexpression of TIMP-2, E-selectin, hsp70 and C-Fn continued to be observed at 24 h. IL-6 was the only cytokine found induced at 24 h. Along with E-selectin, VCAM-1 was another adhesion molecule found to be induced at 24 h. The cardiac specific proteins, cardiac β-myosin and TM, were also found to be induced by Ni at 24 h. V-induced lung injury was associated with increased expression of the chemokine MIP-2, adhesion molecule E-selectin, and C-Fn at 3h, with MIP-2 overexpression continuing even at 24 h (Table 2). ET-1 and EST-1 were two additional messages that were found induced by V at 24 h. No significant exposure specific repression in gene expression was observed among the 84 cardiopulmonary genes assayed in this study.

Verification of the array expression by RT-PCR:

To confirm the differential gene expression observed in the array blot, RT-PCR was carried out on the pooled RNA samples that were utilized for array hybridization. TIMP-2, E-selectin, IL-6, SP-A and β-actin were amplified and analyzed by gel electrophoresis (Fig. 4). Density values of the gene specific DNA bands indicated (Fig. 4, Table 3) that the fold induction in gene expression observed in these selected genes were consistent with array blot.

Discussion

A rat cardiopulmonary cDNA array was developed and utilized in this study to generate expression profiles for rat lungs exposed to a combustion source ROFA and its constituent metals. A comprehensive tissue gene expression profile may be generated by using microarrays containing thousands of genes of known and unknown function. However, our attempt here was to determine whether an expression pattern can be generated using fewer, selected genes, involved in various pathobiological processes of the cardiopulmonary system. The results presented here suggest that studies carried out using a focused gene array may lead to a rational identification of pathological pathway specific gene markers to evaluate potential mechanisms or generate new hypotheses to examine the health effects of complex air pollutants.

Efforts to understand the molecular mechanism(s) underlying the toxicity and adverse health effects of ambient PM have been obscured by several factors such as the complex nature of PM as might relate to regional variability in composition, the extrapolation of animal toxicology data for human health risk assessment, and understanding susceptibility (Kodavanti et al., 1997, 1998b, Bouthillier et al., 1998). Studies carried out in our laboratory and by others using ROFA have demonstrated lung injury and inflammation, and have implicated two major toxic soluble transition metals in ROFA-induced injury (Dreher et al., 1997, Kodavanti et al., 1997, 1998a). Our earlier studies indicated overexpression of certain cytokines and adhesion molecules in rat lungs instilled with ROFA and/or transition metals (Kodavanti et al., 1997, Nadadur et al., 2000). ROFA-induced lung injury and inflammation appear to be mediated in part

by generation of reactive oxygen and nitrogen species that are dependent on its transition metal content. Reactive oxygen species can affect a multitude of signaling pathways involved in regulating gene expression by interacting with promoter elements such as antioxidant response elements (Rushmore et al., 1991) and consensus binding sites for transcription factors like heat shock factor (Bruce et al., 1993), activator protein(AP)-1 (Meyer et al., 1993) and NF-κB (Schreck et al., 1991). Oxidants such as H_2O_2 are known to act like conventional second messenger molecules and impart a high degree of specificity for the regulation of gene expression (Roebuck et al., 1999). *In vitro* studies using rodent (Dye et al., 1999) and human (Quay et. al., 1998, Samet et al., 1999, Silbajoris et al., 2000) airway epithelial cells demonstrated that ROFA and its constituent transition metals induced expression of pro-inflammatory cytokines, transcriptional activation of NF-κB, and stress activated protein kinase. Although these investigations provided altered gene expression data on specific genes using RT-PCR and related methods, it is difficult to gain a comprehensive understanding on the synchronized biological processes involved in ROFA-induced, metal-dependent toxicity and cell injury. A more comprehensive analysis of biological networks implicated in response to toxicant-induced injury is needed to generate and evaluate possible hypotheses on the mechanism(s) of action.

A number of toxicological studies conducted recently to understand PM-induced health effects involved intratracheal and inhalation approaches (Kodavanti et al., 1999, Driscoll et al., 2000). While inhalation exposures are more realistic, intratracheal exposures aid in the initial characterization of toxic responses and also could be used in

selected studies directed to understand mechanism of injury, especially when the test material is available in limited quantities. The dose response relationship derived using IT instillation may differ from inhalation exposures, in terms of gene expression or injury kinetics. While the dose used in the present study for IT instillation was relatively high compared to what might be encountered in ambient air, this dose was needed to identify the early biological pathways implicated in the initial injury response in healthy young adult animals.

The gene expression data obtained in the present study using a focused genearray alone are not sufficient to establish the molecular basis for the interactions among the proteins in a particular metabolic pathway. Simultaneous monitoring of the expression of proteins would be needed to identify specific risk factors implicated in response to a toxicant-induced injury.

The gene expression profile observed in the present study indicates that pulmonary injury caused by ROFA is different from that of its two toxic metals Ni and V. Increased expression of E-selectin in all the groups at 3 h post exposure indicates E-selectin may mediate the early influx of neutrophils to the injury site (Xu et al., 2000) irrespective of the nature of the toxicant or insult. Induction of NOS is known to reduce pulmonary leukocyte accumulation at the site of injury and increased expression of NOS serves as a protective mechanism against lung injury (Kobayashi et al., 2001). Similarly, TIMP-2 is also known to regulate inflammatory lung injury (Gipson et al., 1999). Increased expression of these two messages in ROFA and Ni exposed lung suggest induction of these protective mechanisms. Also, increased expression of IL-6

at 24 h post exposure in ROFA and Ni suggest this cytokine as a common mediator of the inflammatory response in ROFA and Ni-induced injury. Increased expression of TGF-β, PDGF-A, RANTES, IL-5 and HGF by ROFA but not by Ni or V suggests possible involvement of other constituents and constituent interactions responsible for the overall pulmonary injury elicited by ROFA, however, to what extent other constituents may also be involved is unclear. Expression of TGF-β, a known mediator of acute lung injury and the growth factors such as PDGF and HGF suggest induction of proliferative responses and their participation in the extracellular matrix regeneration and/or repair process (Mutsaers et al., 1997, Pitter et al., 2001, Adamson and Bakowska, 2001). Expression of hsp70, C-Fn and VCAM and thrombomodulin only in Ni exposed rats again suggests induction of a systemic thrombogenic response speculated to play a role in cardiac morbidity following PM exposure (Dockery, 2001). The gene expression profile derived for V exposure indicated a different pattern with increased expression of only 3 genes. Consistent increased expression of MIP-2 at 3 and 24 h time points observed in this study suggest MIP-2 to be the critical cytokine mediating V-induced injury and toxicity. V but not Ni-induced expression of MIP-2 has also been reported in rat tracheal epithelial cells (Dye et al., 1999). In these in vitro studies and our earlier in vivo study (Kodavanti et al., 1997) even ROFA was found to induce MIP-2 expression. This expression could be either cell specific or a response to toxic insult as a result of higher dose of ROFA used in the earlier studies. Increased expression of ET-1 by V but not Ni or ROFA exposure suggest that oxidative stress induced by V may also be responsible for the induction of ET-1 expression (Michael et

al., 1997, Love and Keenan, 1998) and subsequently may induce vasoconstriction following PM exposure. Over expression of EST-1 isolated from a carcinogen transformed mouse fibroblast cell line((Nadadur et al., 1997) at 24h post V exposure needs further investigation.

Gene expression profiles generated in the present study led to the following observations: 1). Though water soluble transition metals have been implicated as toxic constituents in ROFA (Dreher et al., 1997, Kodavanti et al., 1997), the toxicity outcome as measured in terms of gene expression profiling suggests that injury from this complex emission PM, ROFA, is different from that of the two major toxic constituents Ni and V suggesting a role for potential constituent interactions. 2). Increased expression of HO-2 by Ni and MIP-2 by V implicate a role for oxidative stress in mediating the inflammatory response (Dennery et al., 1988; Thakker-Varia, et al., 1999; Driscoll, 2000). 3). The difference in the number of cytokines found induced by ROFA compared to Ni and V suggest role for constituent interactions in ROFA toxicity. 4). Genes included in this array were based on their induction in one or more pathological mechanisms and the present observation of their induction confirms their participation in ROFA and metal-induced injury or inflammation. In this process some genes that are down regulated might have been missed.

While pulmonary injury and inflammation are likely due to increased expression of oxidative stress-responsive signaling and subsequent participation of cytokines and chemokines, the gene expression data obtained here also projects a possible role for PM-induced vascular effects (Bouthillier et al., 1998). Increased expression of

thrombomodulin by Ni observed here suggests its possible role in initiating microvascular thrombogenic responses due to influx of neutrophils and subsequent endothelial injury (MacGregor et al., 1997) initiating and propagating signals for vascular thrombosis (Jourdan et al., 1999, Pulido et al., 2000). Similarly increased expression of ET-1 by V also suggest possible alterations in vascular tone and hypertension.

Molecular profiling utilizing a focused genearray can provide basic insights into constituent specific alterations at the gene expression level. Current observations on the induction of specific cytokines by toxic metals provides clues to define the role and participation of genes down-stream of the nuclear transcription factors. Future studies using an advanced version of a more comprehensive cardiopulmonary genearray containing critical functional gene clusters will provide additional leads in identifying constituent specific molecular mechanism(s) for the toxicity of complex air pollutants.

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Legend for Figures :

Fig. 1. Rat cardiopulmonary genearray template indicating spot locations for cDNAs. Refer to Table 1 for details.

Fig.2. Scan images of array blots showing differential gene expression at 3 h following exposure to saline (C) , ROFA (R), nickel (Ni) and vanadium (V). The cDNAs that exhibited differential expression compared to saline treated rat lungs are marked by hollow circles.

Fig. 3. Scan images of array blots showing differential gene expression at 24 h post exposure to saline (C) , ROFA (R), nickel (Ni) and vanadium (V). The cDNAs that are differentially expressed compared to saline treated rat lungs are marked by hollow circles.

Fig. 4. RT-PCR gel analysis for E-selectin, IL-6, TIMP-2, SP-A and β- actin expression following saline (C), ROFA (R), nickel (Ni) and vanadium (V) exposure. Pooled RNA (5 μg) samples that were used for array hybridization were reverse transcribed, amplified in duplicate and analyzed by gel electrophoresis.

Table 1. List of genes grouped in 7 gene clusters of rat cardiopulmonary genearray.

Biological process:	Target gene	Abbreviation
1. Injury/inflammation:	interleukin-1β	IL-1β
	interleukin-5	IL-5
	interleukin-6	IL-6
	interleukin-4	IL-4
	interferon-γ	IFN-γ
	macrophage inflammatory protein-1α	MIP-1α
	macrophage inflammatory protein-2	MIP-2
	monocyte chemoattractant protein-1	MCP-1
	regulated on activation and normal T cell expressed and secreted	RANTES
	tumor necrosis factor-α	TNF-α
	tumor necrosis factor-α receptor 1	TNF-αR1
	transforming growth factor-β	TGF-β
	transforming growth factor-β receptor 1	TGF-βR1
2. Repair/remodeling:	hepatocyte growth factor	HGF
	neuronal growth factor	NGF
	tissue factor	TF
	tissue factor inhibitor	TFI
	platelet derived growth factor ligand A	PDGF-A
	platelet derived growth factor ligand B	PDGF-B
	platelet derived growth factor receptor-α	PDGF-α
	platelet derived growth factor receptor-β	PDGF-β
	platelet activating factor	PAF
	platelet activating factor receptor	PAF-R
	vascular endothelial growth factor-D	VEGF-D
	E-selectin	E-sel.
	P-selectin	P-sel.
	intracellular adhesion molecule-1	ICAM-1
	vascular cell adhesion molecule-1	VCAM-1
	cellular fibronectin EIII-A	C-Fn
	surfactant protein-A	SP-A
	surfactant protein-D	SP-D
	β-actin	β-actin
	troponin	Tropo.
	clusterin	Clust.
	α-fibrinogen	α-fibri.
	γ-fibrinogen	γ-fibri.
	cardiac β-myosin	Card.β-myo.
	cardiotroponin	Cardiotropo.
	K-kininogen	K-kinino.
	α-actin cardiac protein	AACP

Table 1. Continued

Biological process:	Target gene	Abbreviation
3. Stress response:	heat shock protein 70	hsp70
	major acute phase protein	MAPP
	protein-C	Prot.C
	heme oxygenase-2	HO-2
	manganese dependent super oxide dismutase	MnSOD
	tissue inhibitor of mettalloproteinase-1	TIMP-1
	tissue inhibitor of mettalloproteinase-2	TIMP-2
4.Vascular function:		
	endothelin-1	ET-1
	endothelin-1 receptor A	ET-1A
	endothelin-1 receptor B	ET-1B
	endothelin converting enzyme	ECE
	angiotensinogen	Ang.
	angotensin II converting enzyme	ACE
	angiotensin-II receptor type 1A	Ang.II 1A
	angiotensin-II receptor type 2 A	Ang.II 2A
	atrial natriuretic factor	ANF
	kinase insert domain containing receptor	KDR
	thrombomodulin	TM.
	renin	Renin
	c-raf	C-raf
	nitric oxide synthase	NOS
	induced nitric oxide synthase	NOS(I)
5. Kinases :		
	extracellular signal regulated kinase-7	ERK7
	mitogen activated protein kinase p38	MAPK
	fms-like tyrosine kinase	Fms-TK
	protein kinase C-α	PKC-α
	protein kinase C-α1	PKC-α1
	protein kinase C-β	PKC-β
	stress activated protein kinase-α	SAPK-α
	cyclin dependent kinase-2	CDK2
	chp-Janus kinase	Chp-JNK
6. Oncogenes/Transcription factors:	ras oncogene H-ras	Ha-ras
	c-fos	C-fos
	bcl-2	bcl-2
	nuclear factor kappa B	NF-κB
	tumor suppressor protein 53	p53
	p21 cip1/Waf1	p21
	Cyclin E	Cyclin-E

Table 1. Continued

Biological process:	Target gene	Abbreviation
7. Energy metabolism :	glyceraldehyde 3-phosphate dehydrogenase	G3PDH
	sodium-potassium dependent ATPase	N/K ATPase
	calcium-dependent ATPase	Ca-ATPase
Miscellaneous :	monoamine oxidase	MAO
	rat liver male specific P-450	SK-15
Expressed sequence tags :	ESTs isolated from mouse cell line	EST-1, 3, 5, 6
	Long terminal repeat seq from rat	SK72
	EST isolated from human cell line	EST-2, 4

Table2. Fold induction in the expression of genes observed in the genearray following exposure to ROFA, Ni and V.

Coordinate with abbreviation	ROFA 3h	ROFA 24h	Ni 3h	Ni 24h	V 3h	V 24h
A2 : MIP-2					1.8*	2.2
A3: HGF		1.6				
A4: E-sel	1.7	2.0	2.7	2.1	1.9	
A6: HO-2			2.8			
A8: ET-1						1.8
A11: Card.β-myo				1.9		
B2: RANTES		1.8				
B4: C-Fn			1.9	2.0	1.9	
B6: hsp70			1.8	1.9		
C1: IL-5		1.8				
C4: ICAM-1		1.9	1.7			
C6: MAPP	2.2					
D1: IL-6		2.4		1.8		
E2: PDGF-A	2.4	2.2				
E4: VCAM-1				2.1		
E6: NOS	2.7					
F3: TGF-β	1.8	1.8				
F6: NOS(I)	2.1		2.1			
F10: TM				2.0		
H4: EST-1						2.5
H6: TIMP-2	2.1	1.9	2.3	1.8		

* The fold difference in the expression was calculated using mean of two independent hybridization experiments carried out in duplicate using pooled RNA samples.

Table 3. Densitometric units for RT-PCR amplified cDNA bands detected on agarose gel.

Target Gene	3h				24 h			
	C	R	Ni	V	C	R	Ni	V
E-Selectin	1,265	3,549	4,864	2,788	1,480	4,496	4,087	1,714
IL-6	834	896	758	865	939	2,712	2,055	748
TIMP-2	5,816	14,881	12,780	6,823	8,469	20,878	17,380	7,992
SP-A	85,663	78,757	81,389	74,546	91,400	79,540	76,823	74,692
β- actin	66,484	66,388	61,379	60,878	60,580	60,724	54,887	51,798

* The values indicate integrated density volume that is automatically corrected for background. Each value is an average of two samples in duplicate.